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Regulation of Pyruvate Dehydrogenase Kinase 4 (PDK4) by CCAAT/Enhancer-binding Protein β (C/EBPβ)*[S]

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The conversion of pyruvate to acetyl-CoA in mitochondria is catalyzed by the pyruvate dehydrogenase complex (PDC). Activity of PDC is inhibited by phosphorylation via the pyruvate dehydrogenase kinases (PDKs). Here, we examined the regulation of *Pdk4* gene expression by the CCAAT/enhancer-binding protein β (C/EBPβ). C/EBPβ modulates the expression of multiple hepatic genes including those involved in metabolism, development, and inflammation. We found that C/EBPβ induced *Pdk4* gene expression and decreased PDC activity. This transcriptional induction was mediated through two C/EBPβ binding sites in the *Pdk4* promoter. C/EBPβ participates in the hormonal regulation of gluconeogenic genes. Previously, we reported that *Pdk4* was induced by thyroid hormone (T_3). Therefore, we investigated the role of C/EBPβ in the T_3 regulation of *Pdk4*. T_3 increased C/EBPβ abundance in primary rat hepatocytes. Knockdown of C/EBPβ with siRNA diminished the T_3 induction of the *Pdk4* and carnitine palmitoyltransferase (*Cpt1a*) genes. CPT1a is an initiating step in the mitochondrial oxidation of long chain fatty acids. Our results indicate that C/EBPβ stimulates *Pdk4* expression and participates in the T_3 induction of the *Cpt1a* and *Pdk4* genes.

Regulation of gene expression by steroid hormones involves the interaction of transcription factors, nuclear receptors, and coactivators (1). In this study, we investigated the role of the CCAAT/enhancer-binding protein β (C/EBPβ)[4] in the regulation of pyruvate dehydrogenase kinase (PDK) and carnitine palmitoyltransferase (CPT1a) genes. C/EBPs constitute a family of transcription factors with multiple members including C/EBPα, C/EBPβ, and others (2, 3). C/EBP isoforms share two highly conserved domains, the C-terminal basic DNA binding domain and the leucine zipper domain as well as the less homologous N-terminal activation domain (4). C/EBPβ is highly expressed in liver, adipose tissue, intestine, lung, and others (3, 4). In the liver, C/EBPβ stimulates genes encoding gluconeogenic enzymes including phosphoenolpyruvate carboxykinase (PEPCK) (5–7) as well as genes involved in hepatic lipogenesis such as fatty acid synthase (FASN) and acetyl-CoA carboxylase (8, 9). Insulin and glucocorticoids regulate transcription of C/EBPβ and the binding of C/EBPβ alternate translation proteins to gluconeogenic genes (10–12).

Thyroid hormone (T_3) plays an important role in various aspects of metabolism and development (13). The genomic actions of T_3 are mediated through the binding of thyroid hormone receptors (TRs) to T_3-response elements (TREs) (14). Two TR isoforms (TRα and TRβ) are encoded on separate genes with TRβ being the more abundantly expressed isoform in the liver (14). Liganded TR stimulates transcription through the recruitment of various coactivators as well as via interactions with other transcription factors (1, 15). T_3 increases the expression of genes involved in hepatic fatty acid oxidation, especially *Cpt1a* (16). T_3 elevates hepatic triglyceride production as well as a range of genes involved in hepatic lipogenesis and low density lipoprotein receptor expression (17–19). With respect to glucose metabolism, T_3 stimulates gluconeogenesis by elevating the transcription of the gluconeogenic enzymes, glucose-6-phosphatase and PEPCK (20–22).

It was observed by Menéndez-Hurtado *et al.* (23) that hypothyroidism in pregnant rats led to a decrease in both C/EBPα and C/EBPβ hepatic gene expression in the pups during postnatal development. In addition, the hypothyroid neonatal pups had diminished C/EBPα and C/EBPβ protein levels. This reduction in C/EBP expression was confined to the liver because C/EBP mRNA levels in brown fat were unchanged. Injection of hypothyroid animals with T_3 resulted in the recovery of C/EBPα and C/EBPβ mRNA levels in the liver. Studies from our laboratory showed that C/EBPα was needed for the induction of *Pepck* by T_3 (24). Other investigators have shown that C/EBPα participates in the actions of T_3 in liver, kidney, and brown adipose tissue (25–27). The C/EBPβ isoform has not been examined with respect to its role in T_3 action. Therefore, we investigated the role of C/EBPβ in induction of *Pdk4* and *Cpt1a* by T_3.

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[4] The abbreviations used are: C/EBPβ, CCAAT/enhancer-binding protein β; PDC, dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; T_3, thyroid hormone; TR, thyroid hormone receptor; TRE, T_3-response element; PEPCK, phosphoenolpyruvate carboxykinase; CREB, cAMP-response element-binding protein; FP, forward primer; RP, reverse primer; Ad, adenovirus.

The pyruvate dehydrogenase complex (PDC) catalyzes the oxidative decarboxylation of pyruvate into acetyl-CoA. The phosphorylation of PDC on three serine residues of the E1α subunit by PDK inhibits PDC activity (28, 29). The abundance of the PDK4 isoform is transcriptionally controlled (30). Expression of the *Pdk4* gene is increased by T_3, glucocorticoids, retinoic acid, and long chain fatty acids, whereas it is inhibited by insulin (30, 31). We recently identified two TREs in the *Pdk4* gene promoter and demonstrated that the peroxisome proliferator-activated receptor γ coactivator (PGC-1α) enhances the T_3 induction of the *Pdk4* expression (32). CPT1a catalyzes the transfer of fatty acids from long chain acyl-CoA to carnitine and is an initiating step in the translocation of long chain fatty acids across the mitochondrial membranes (33). PGC-1α also enhances the induction of *Cpt1a* by T_3 (34).

In this study, we characterized the role of C/EBPβ in the regulation of *Pdk4* gene expression. Our data indicate that C/EBPβ strongly stimulates the expression of the *Pdk4* gene through multiple sites in the promoter. In addition, C/EBPβ participates in the induction of *Pdk4* expression by T_3, and the abundance of C/EBPβ is elevated by T_3. Moreover, C/EBPβ is involved in the T_3 stimulation of the *Cpt1a* gene, suggesting that C/EBPβ can affect the hormonal regulation of genes involved in glucose and fatty acid metabolism.

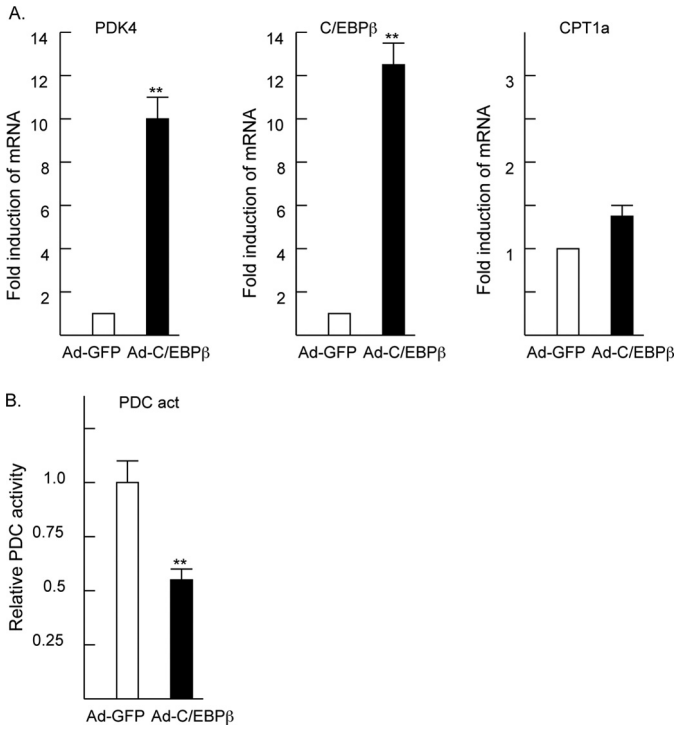

FIGURE 1. **C/EBPβ stimulates PDK4.** *A*, McA-RH777 cells were infected with adenoviruses expressing either GFP (Ad-GFP) or C/EBPβ (Ad-C/EBPβ). After 40 h, the cells were harvested, and C/EBPβ, *Pdk4*, and *Cpt1a* mRNA abundance was assessed by real-time PCR. The data are expressed as -fold induction of mRNA. *B*, McA-RH7777 cells were infected with Ad-GFP or Ad-C/EBPβ as described above. The cells were harvested, and the PDC activity was assessed using the MitoSciences MSP30 assay kit for PDC activity (*PDC act*) as outlined under "Materials and Methods." Relative quantification of the assay activity was performed using the Alpha View SA software. All experiments were repeated 4 times. **, $p = 0.001–0.01$.

MATERIALS AND METHODS

Transient Transfection of Luciferase Vectors—*Pdk4*-luciferase constructs (*Pdk4*-luc) were transiently transfected into HepG2 cells by the calcium phosphate method as described previously (34). *Pdk4*-luc was transfected with the expression vectors SV40-TRβ, MSV-C/EBPβ, and TK-*Renilla*. Cells were transfected in DMEM containing 5% calf serum and 5% FCS. After overnight incubation, the medium was replaced by DMEM without serum, and the cells were treated with 100 nM T_3 for 24 h. Transfected cells were harvested in passive lysis buffer (Promega, Madison, WI). Luciferase and *Renilla* luciferase activity was measured with the Promega Dual-Luciferase reporter kit (E1980). Protein content in each lysate was determined by Pierce BCA protein assay kit (Thermo Scientific). Luciferase activity was corrected for both protein content and *Renilla* luciferase activity.

Mutagenesis of Pdk4 Promoter—Serial deletions of the rat *Pdk4* promoter were created by PCR amplification as described previously (35). The QuikChange XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used to alter the C/EBPβ sites in the *Pdk4*-luciferase vector. The following forward primers (FPs) and reverse primers (RPs) were used to introduce these mutations: −96 mutant FP, 5′-gctctgagctgattggtgtgttttcaagttcagagtcccagag-3′; −96 mutant RP, 5′-ctctgggactctgaacttgaaaacacaccaatcagctcagagc-3′; −629 mutant FP, 5′-aaaaaaccggtagaaagtgttctgagggagaagtgaactggc-3′; −629 mutant RP, 5′-gcccagttcacttctccctcagaacactttctaccggtttttt-3′.

Electrophoretic Mobility Shift Assay—To conduct electrophoretic mobility shift assays, double-stranded oligonucleotides were labeled with Klenow enzyme and [α-^{32}P]dCTP (16). Oligonucleotides contained sequences representing the C/EBPβ sites (see Fig. 2). Nuclear proteins from rat liver were obtained by the method of Gorski *et al.* (36). The protein-DNA binding mixtures contained labeled probe and nuclear proteins in 80 mM KCl, 25 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and poly(dI·dC). The binding reactions were incubated at room temperature and resolved on 5% acrylamide gels (80:1 acrylamide/bisacrylamide) in running buffer containing 22 mM Tris and 190 mM glycine (16).

Western Analysis—Western analysis was performed on whole cell extracts from primary rat hepatocytes (37). Hepatocytes were lysed in radioimmune precipitation buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% deoxycholate, 5 mM EDTA, pH 8.0, 0.1% SDS, and diluted protease inhibitor mixture). The cells were vortexed for 1 min, and cell membranes were removed by centrifugation for 25 min at 4 °C. An equal amount of protein was loaded on a 12% SDS-PAGE gel and transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad). The membranes were immunoblotted with primary antibodies C/EBPβ (sc-150, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS containing 5% nonfat dry milk powder and were incubated with HRP-conjugated anti-rabbit secondary antibody. Immunoreactive proteins were detected using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific). The ChemiDoc™ XRS gel documentation system (Bio-Rad) was used to quantify the immunoreactive proteins. β-Actin (sc-1615, Santa Cruz Biotechnology) was used as the loading control for each lane.

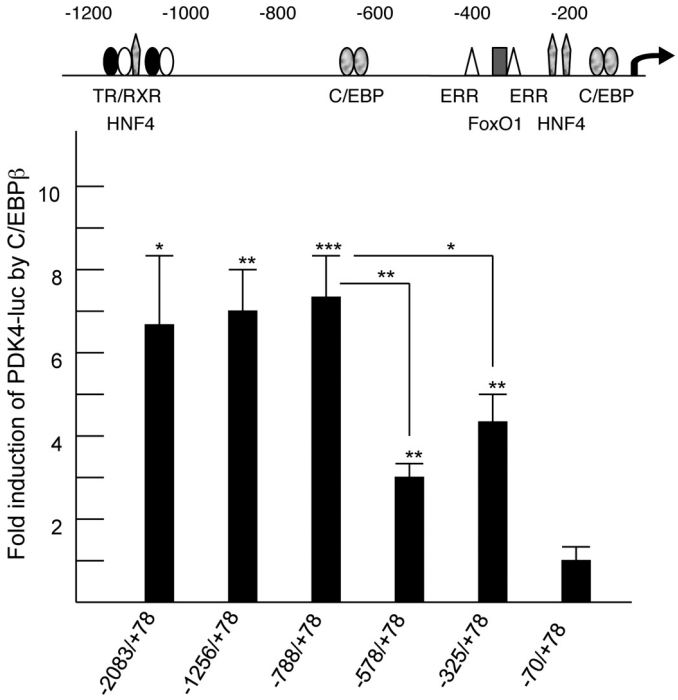

FIGURE 2. **C/EBPβ increases PDK4 promoter activity.** A model of the *Pdk4* promoter is shown with known factor binding sites schematized. The TRβ and HNF4 sites are overlapping. HepG2 cells were transiently transfected with 2 μg of rat *Pdk4* luciferase reporters, 1 μg of MSV-C/EBPβ, and 0.1 μg of TK-*Renilla*. All transfections were performed in duplicate and repeated 4–6 times. Luciferase activity was corrected for both protein content and *Renilla* luciferase activity. Results are expressed as the -fold induction of *Pdk4* by C/EBPβ ± S.E. by comparing the induction of vectors in the C/EBPβ overexpressing cells with the controls. The significance is calculated relative to the shortest construct of the rat *Pdk4*-luciferase (−70/+78) (*, $p = 0.01–0.05$, **, $p = 0.001–0.01$, ***, $p < 0.001$). *RXR*, retinoid X receptor; *ERR*, estrogen related receptor.

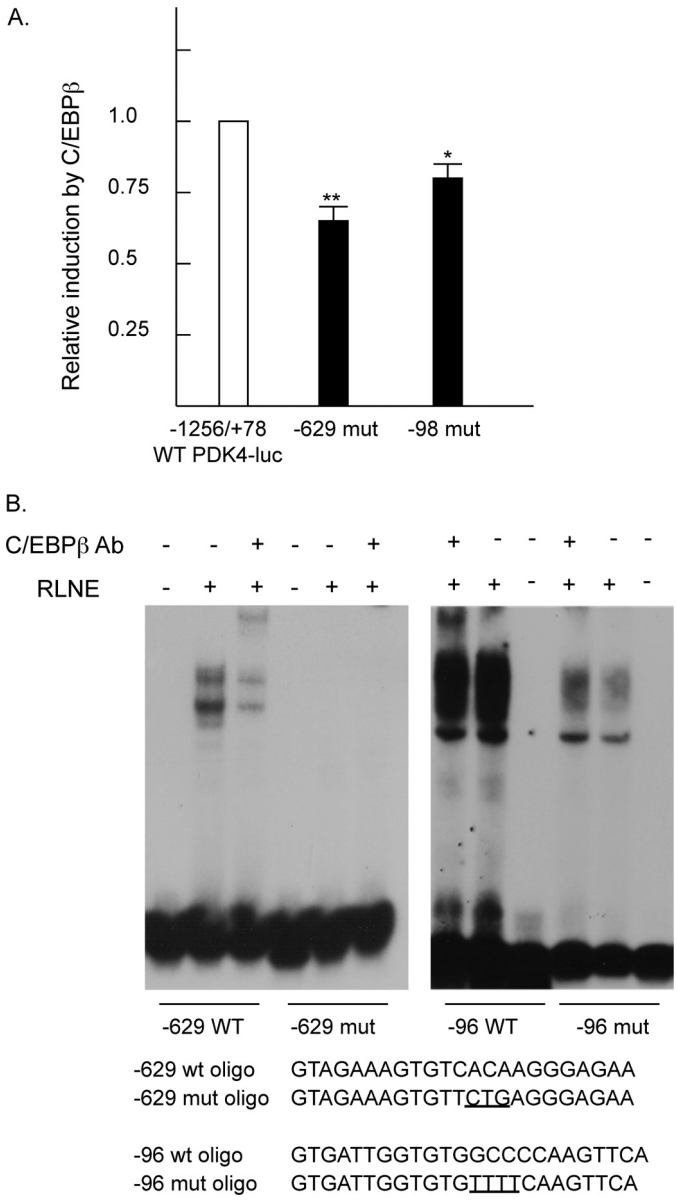

FIGURE 3. **Identification of C/EBPβ binding sites in the rat *Pdk4* promoter.** *A*, HepG2 cells were transiently transfected with −1256/+78 rat *Pdk4*-luciferase, MSV-C/EBPβ, and TK-*Renilla*. The potential C/EBPβ binding sites were disrupted. All transfections were performed in duplicate and repeated 4 times. Results are expressed as the relative percentage of induction of *Pdk4* by C/EBPβ ± S.E. Significance is calculated relative to the wild type rat *Pdk4* −1256/+78 luciferase (*, $p = 0.01–0.05$, **, $p = 0.001–0.01$). *B*, C/EBPβ binding to the promoter sites was tested with gel shift mobility assays. Gel shift assays were conducted as described under "Materials and Methods." Sequences for the wild type C/EBPβ binding sites (−96) and (−629) oligomer (*wt oligo*) or the mutated version (*mut*) are shown below the gel shift with the mutated nucleotides *underlined*. *Ab*, antibody; *RLNE*, rat liver nuclear extract.

Determination of PDC Activity—The PDC activity was measured with the Dipstick assay kit from MitoSciences (MSP30, Eugene OR). The validity of the assay has been demonstrated previously (38, 39). Cells transduced with 50 pfu/cell Ad-C/EBPβ or Ad-GFP were scraped from plates in the provided sample buffer and pelleted by centrifugation. Following the kit protocol, equal amounts of protein were added to plate wells, and dipsticks were inserted into the wells. The intensity of the bands representing active PDC was quantified densitometrically using Alphaimager EP and AlphaView SA software (Cell Biosciences, Santa Clara, CA).

Real-time PCR—The cDNA for real-time PCR was prepared using RNA isolated from primary rat hepatocytes. RNA was isolated with RNA-Stat-60 (Tel-test, Friendswood, TX) (40). The RNA was treated with DNase I (2 units) at 37 °C for 1 h followed by the addition of DNase inactivation reagent (Ambion, Austin, TX), and the concentration of each sample was measured using NanoDrop (Thermo Scientific). Two μg of DNA-free RNA was converted to cDNA using SuperScript reverse transcriptase III and random hexamers (Invitrogen). The parameters for real-time PCR were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 30 s and 60 °C for 1 min. The final concentration of primers in each well in the PCR plates was 0.1 μM. The target genes were normalized with the 18S gene. The following FPs and RPs were used for real-time PCR: PDK4 FP, 5′-ggattactgaccgcctctttagtt-3′; PDK4 RP, 5′-gcattccgtgaattgtccatc-3′; CPT1a FP, 5′-cggttcaagaatggcatcatc-3′; CPT1a RP, 5′-tcacacccaccaccacgat-3′; 18S FP, 5′-cggctaccacatccaaggaa-3′; 18S RP, 5′-ttttcgtcactacctccccg-3′. Primers for C/EBPβ and FASN were obtained from Qiagen (41) (QT00366478 and QT00371210, respectively).

Chromatin Immunoprecipitation Assays—ChIP assays were conducted with modifications following the protocol given by the Millipore Magna ChIP kit (17-610) (42). Rat primary hepatocytes were maintained for 18 h in RPMI 1640 medium containing 5% fetal bovine serum and 5% calf serum. Cells were

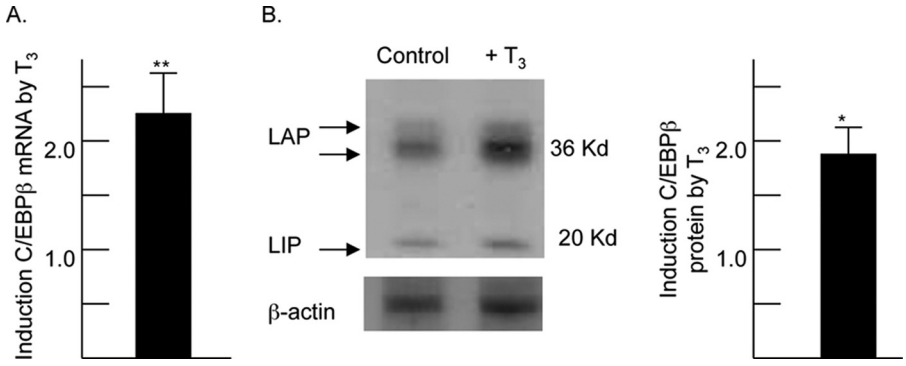

FIGURE 4. **Thyroid hormone increases C/EBPβ protein abundance.** Primary rat hepatocytes were plated on collagen-coated plates, and T_3 was added at a concentration of 100 nM for 24 h. *A*, induction of C/EBPβ mRNA abundance is shown. The data are presented as the -fold induction of mRNA abundance by T_3 (average ± S.E.) from four independent preparations of hepatocytes. *B*, the expression of the C/EBPβ protein was monitored by Western blot analysis. The -fold induction of C/EBPβ abundance by T_3 was assessed from four hepatocyte preparations. The control samples were assigned a relative value of 1 (*, p = 0.01–0.05, **, p = 0.001–0.01). *LAP*, liver-enriched activator protein; *LIP*, liver-enriched inhibitory protein.

treated with 100 nM T_3 overnight in serum-free medium. After treatment, cross-linking was performed with 1% formaldehyde for 10 min at room temperature, and cells were sonicated as described previously (42). Chromatin preparations were diluted with dilution buffer and protease inhibitor mixture and designated as input samples (no antibody) or immunoprecipitated with the control antibody rabbit IgG (sc-2027, Santa Cruz Biotechnology), anti-C/EBPβ (sc-150, Santa Cruz Biotechnology), or anti-TRβ (MA1-216, Thermo Scientific). Samples were mixed overnight at 4 °C with the antibody and magnetic protein A beads. The beads were washed, and the DNA was eluted. Eluted DNA was purified using the PCR purification kit (Qiagen 28104). DNA was subjected to 35 cycles of PCR using 3–6 μl of DNA. PCR products were analyzed on 2% NuSieve 3:1 agarose (Lonza, Walkersville, MD) and visualized with Multi-Image light cabinet with Alphaimager EP software. The following primers were used to amplify portions of the *Pdk4* promoter: the proximal *Pdk4* promoter (−591/−338) FP 5′-taaggctatttaggcagttt-3′ and RP 5′-ccagacttgtccttgtttac-3′, the TRE (−1535/−1228) FP 5′-agtgtctccaccagattgt-3′ and RP 5′-ctaagagagctaacctagt-3′, and the upstream region (−6634/−6377) FP 5′-tatgagaagtgctgcaataa-3′ and RP 5′-atgttaccacaa-accttcat-3′ (42).

Knockdown of C/EBPβ in Hepatocytes—Adenoviruses encoding shRNA specific for C/EBPβ (Ad-siC/EBPβ) and control adenoviruses encoding non-template shRNA were constructed as described previously (43). Hepatocytes were plated at a density of 3×10^6 in a 60-mm dish in RPMI 1640 medium. The adenoviruses were added at a multiplicity of infection of 50. Media were changed 24 h after transduction, and the cells were treated with 100 nM T_3 for another 24 h in serum-free RPMI 1640 before harvesting the cells.

RESULTS

Induction of Pdk4 by C/EBPβ—Previous studies from our laboratory and others found that C/EBPβ participates in both the basal expression and the hormonal regulation of gluconeogenic genes (6, 10, 44). To test whether C/EBPβ would induce the *Pdk4* gene, we overexpressed C/EBPβ in McA-RH7777 hepatoma cells by adenoviral infection. C/EBPβ increased the abundance of *Pdk4* mRNA 9.8-fold (Fig. 1*A*). The activity of PDC was decreased 48%, suggesting that the increase in PDK4 was inhibiting PDC (Fig. 1*B*). In addition, we measured *Cpt1a* mRNA as fatty acid oxidation is often increased as glucose oxidation is diminished. *Cpt1a* mRNA abundance was increased slightly (Fig. 1*A*).

Identification of C/EBPβ-responsive Elements in the Rat Pdk4 Promoter—To investigate the ability of C/EBPβ to induce the *Pdk4* promoter, we cotransfected serial deletions of the rat *Pdk4* promoter driving a luciferase reporter with or without MSV-C/EBPβ in HepG2 hepatoma cells and measured luciferase activity. Deletion of 210 nucleotides between −788 and −578 of the rat *Pdk4* promoter reduced C/EBPβ responsiveness from 7.6 ± 0.7-fold to 3.3 ± 0.5-fold. In addition, deletion of the 255 nucleotides between −325 and −70 of the rat *Pdk4* promoter diminished C/EBPβ responsiveness from 4.4 ± 0.8- to 1.00 ± 0.3-fold (Fig. 2). These data indicate that C/EBPβ induced *Pdk4* gene expression through two regions in the promoter.

To identify specific C/EBPβ-response elements, we conducted site-directed mutagenesis of putative C/EBP binding sites in the −1256/+78 *Pdk4*-luciferase vector (*Pdk4*-luc). Mutation of two C/EBPβ elements, localized between −634 to −626 and −96 to −87, decreased the ability of C/EBPβ to induce the *Pdk4* gene (Fig. 3*A*). Gel shift mobility assays were conducted to determine whether C/EBPβ could bind these sites. Oligomers were incubated with rat liver nuclear extract and antibodies to C/EBPβ. In each case, a supershift was observed indicating that C/EBPβ bound the site (Fig. 3*B*). Multiple proteins were able to bind to the −96 to −87 site as the C/EBPβ antibody supershifted only a small amount of the DNA-protein complex.

T_3 Increases C/EBPβ Abundance in Hepatocytes—We next investigated whether T_3 treatment could induce C/EBPβ expression. Primary rat hepatocytes were treated with 100 nM T_3 for 24 h. C/EBPβ mRNA abundance was elevated 2.2 ± 0.4-fold (Fig. 4*A*). C/EBPβ protein abundance was increased 1.8 ± 0.3-fold in response to T_3 treatment (Fig. 4*B*). This experiment demonstrated that the C/EBPβ is a T_3-responsive gene and raised the possibility that it might participate in the regulation of the *Pdk4* gene by T_3.

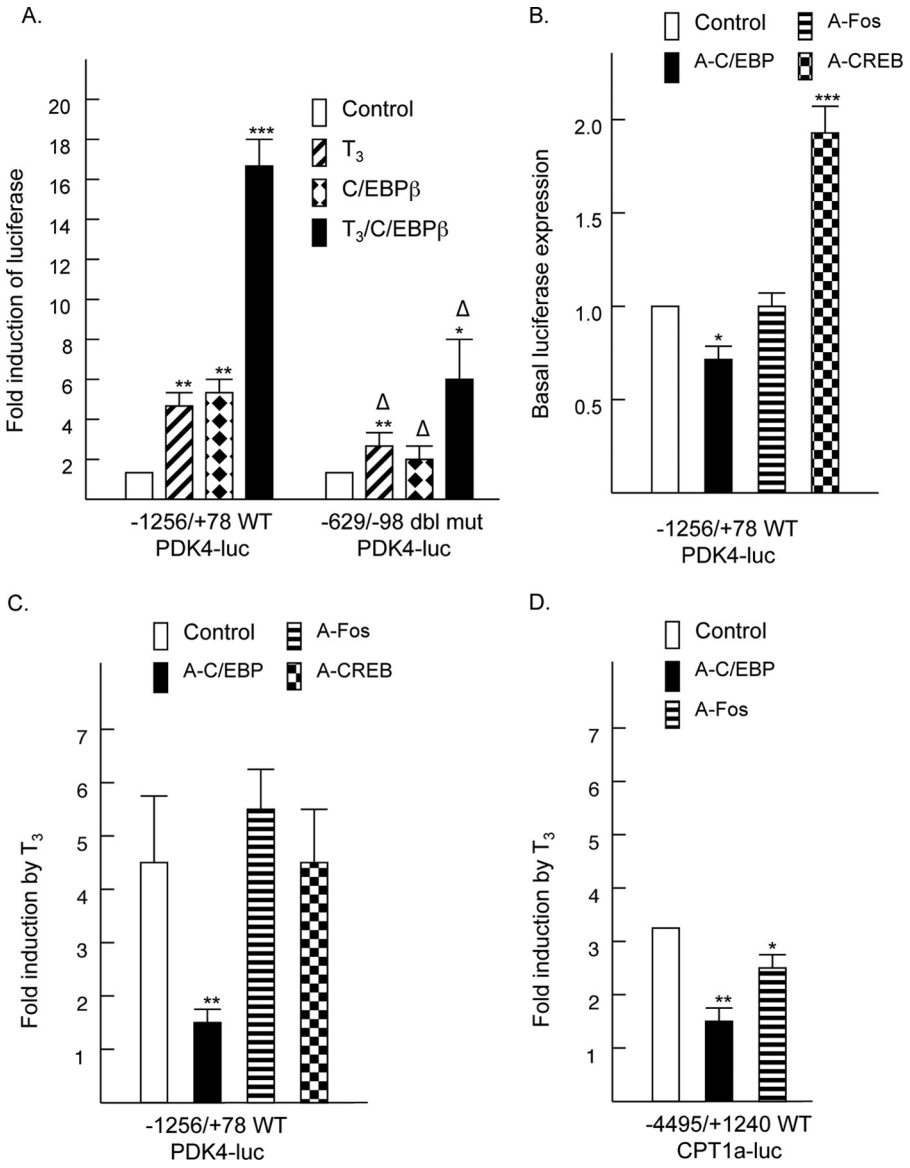

FIGURE 5. **C/EBPβ enhances the T_3 induction of rat *Pdk4* gene.** *A*, overexpression of C/EBPβ enhances T_3 induction of *Pdk4*-luc. HepG2 cells were transfected with wild type or −629/−96 C/EBP double mutant (*dbl mut*) *Pdk4*-luc. Also included were MSV-C/EBPβ, SV40-TRβ, and TK-*Renilla*. T_3 was added at a concentration of 100 nM for 24 h. The data are expressed as -fold induction of luciferase (*, $p = 0.01–0.05$, **, $p = 0.001–0.01$, ***, $p < 0.001$, Δ, p value 0.001–0.01 between −1256/−78 rat *Pdk4* wild type and the corresponding treatment for the −629/−96 double mutant rat PDK4). *B*, HepG2 cells were transfected with PDK4-luc and dominant negative expression vectors for C/EBP (A-C/EBP), CREB (A-CREB), or Jun (A-Fos). Results are expressed as the basal level compared with cells without dominant negative vectors. *C*, A-C/EBP abolished the ability of T_3 to induce *Pdk4*-luc. *D*, A-C/EBP decreased T_3 induction of *Cpt1a*-luciferase. For *C* and *D*, the results are expressed as -fold induction by T_3 (*, $p = 0.01–0.05$, **, $p = 0.001–0.01$). All transfections were performed in duplicate and repeated 3–6 times.

C/EBPβ Enhances the T_3 Induction of the Pdk4 Gene—To investigate whether C/EBPβ participated in the T_3 induction of *Pdk4*, we cotransfected wild type −1256/+78 *Pdk4*-luc and MSV-C/EBPβ into HepG2 cells with or without T_3. The addition of 100 nM T_3 stimulated wild type −1256/+78 *Pdk4*-luc 4.7 ± 1-fold. In these experiments, overexpression of C/EBPβ induced the *Pdk4*-luc vector 5.3 ± 1-fold. When T_3 was added in the presence of C/EBPβ, a synergistic 16.9 ± 2-fold induction was observed (Fig. 5*A*). These data indicate that C/EBPβ can activate the *Pdk4* gene promoter and amplify the T_3 effect on the *Pdk4* promoter. In addition, we tested the *Pdk4*-luc vector in which both C/EBP binding sites were disrupted (Fig. 5*A*). This vector was modestly induced by C/EBPβ, suggesting that an additional C/EBPβ binding site is present in the promoter. The T_3 induction was reduced in the vector with disrupted C/EBPβ binding sites, indicating that C/EBPβ participates in the T_3 action.

To further investigate the role of C/EBPβ in the induction of *Pdk4* transcription, we tested a set of dominant negative vectors. The dominant negative C/EBP vector, A-C/EBP, has the C/EBP leucine zipper attached to an acidic amphipathic helix. This helix interacts with the basic region of C/EBP proteins to form a non-DNA binding heterodimer. For controls, we used A-CREB, which is a dominant negative CREB protein (45), and the dominant negative Jun vector A-Fos (45). The −1256/+78 *Pdk4*-luc vector was cotransfected with the dominant negative vectors into HepG2 cells, and luciferase activity was assessed. A-C/EBP reduced basal

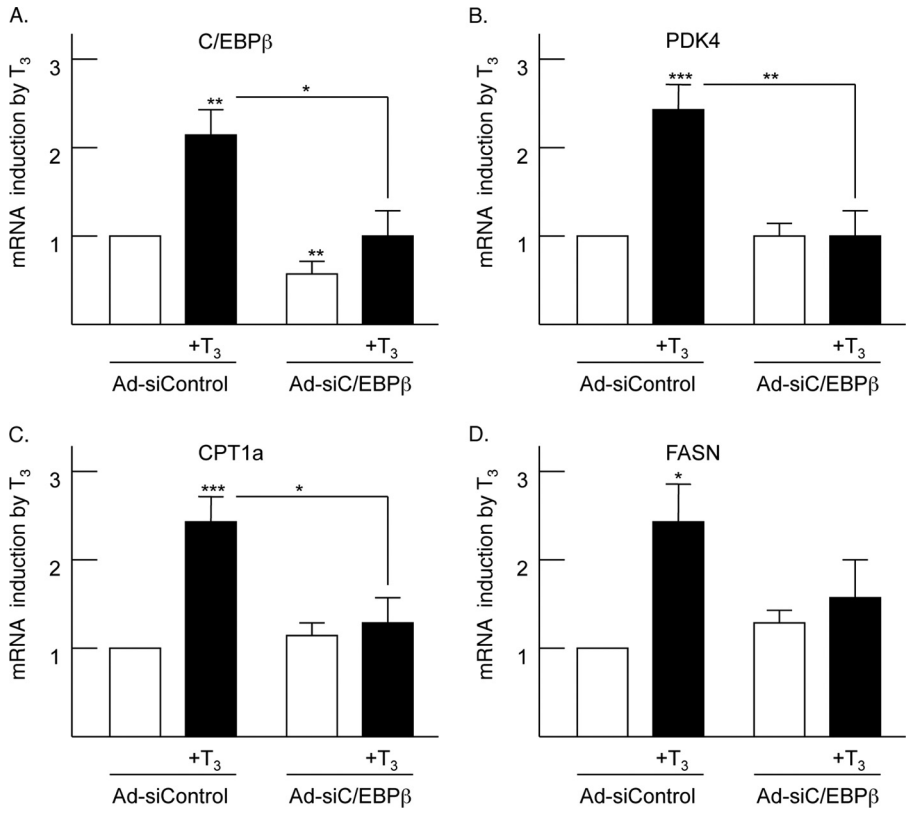

FIGURE 6. **C/EBPβ knockdown reduces T_3 responsiveness.** Rat primary hepatocytes were infected with adenoviral vectors expressing shRNA for C/EBPβ (Ad-siC/EBPβ) or expressing control-shRNA (Ad-siControl). Hepatocytes were treated with T_3 at a concentration of 100 nM for 24 h. The mRNA abundance of the indicated genes was measured by real-time PCR. The infections were repeated 3 or 4 times on independent preparations of hepatocytes. *A*, -fold induction of the C/EBPβ gene expression in comparison with hepatocytes infected with Ad-siControl. *B–D*, the abundance of the following mRNAs was also measured: *Pdk4* (*B*); *Cpt1a* (*C*); and *Fasn* (*D*). The data are expressed as the mean of the -fold induction by T_3 ± S.E. of mRNA abundance relative to untreated cells (*, p = 0.01–0.05, **, p = 0.001–0.01, ***, $p < 0.001$).

expression of the *Pdk4* gene by 32% (p = 0.02), whereas the A-Fos did not affect the basal level of the *Pdk4* gene. A-CREB increased the *Pdk4*-luciferase activity (p = 0.0007) (Fig. 5*B*), although the reason for this increase is not clear. In addition, A-C/EBP abolished the ability of T_3 to induce *Pdk4*-luciferase (Fig. 5*C*). In previous studies, we demonstrated that C/EBPα bound to the intron of the *Cpt1a* gene (46). *Cpt1a* is a T_3-responsive gene, so we examined the effect of A-C/EBP on the T_3 induction of *Cpt1a*. A-C/EBP reduced the T_3 induction of *Cpt1a*-luciferase, suggesting that C/EBPβ is important in the T_3 responsiveness of multiple genes (Fig. 5*D*).

Knockdown of C/EBPβ Reduces Pdk4 Gene Expression in Primary Rat Hepatocytes—To examine the role of the C/EBPβ in the T_3 induction of the endogenous *Pdk4* gene, we infected rat primary hepatocytes with adenoviruses encoding shRNA to silence C/EBPβ (Ad-siC/EBPβ). The hepatocytes were infected for 16 h prior to the addition of T_3. Adenovirus encoding control shRNA that does not silence any rat genes was used as a control (Ad-siControl) (43). Ad-siC/EBPβ knocked down the C/EBPβ mRNA abundance by 50%. In addition, the induction of C/EBPβ by T_3 was reduced in hepatocytes infected with Ad-siC/EBPβ in comparison with the 2.2 ± 0.4-fold induction of the T_3-treated control cells (Fig. 6*A*). Knockdown of C/EBPβ in primary hepatocytes decreased the T_3 induction of PDK4 as T_3 induced *Pdk4* only by 1.2 ± 0.2-fold, whereas T_3 increased *Pdk4* in hepatocytes infected with Ad-siControl by 2.4 ± 0.2-fold (Fig. 6*B*). This inhibition was also observed with the *Cpt1a* gene expression as T_3 failed to induce *Cpt1a* following C/EBPβ knockdown (Fig. 6*C*). Although the T_3 induction of *Fasn* was decreased by C/EBPβ knockdown, the decrease did not reach statistical significance. These data indicate that C/EBPβ is an important coregulator in the T_3 induction of the *Pdk4* and *Cpt1a* genes.

Next, we tested whether T_3 altered the binding of C/EBPβ to the *Pdk4* gene. ChIP assays were conducted on the PDK4 promoter following T_3 treatment. We observed C/EBPβ association with the *Pdk4* promoter, but C/EBPβ binding was not elevated, suggesting that T_3 does not increase C/EBPβ association with the promoter (Fig. 7*B*). As a control to test whether we could observe elevated C/EBPβ binding, we overexpressed C/EBPβ by adenoviral infection and conducted ChIP assays. Under these conditions, we observed a 2-fold enhancement of C/EBPβ association with the *Pdk4* promoter (supplemental Fig. 1). These data suggested that the 2-fold induction of C/EBPβ protein by T_3 might not generate sufficient increase in binding to be quantified in our ChIP assays at least at the time point at which the cross-linking was conducted. We also tested whether the knockdown of C/EBPβ would result in decreased TRβ recruitment to the *Pdk4* promoter. Hepatocytes were infected with Ad-siC/EBPβ. Our ChIP assay results indicated that the

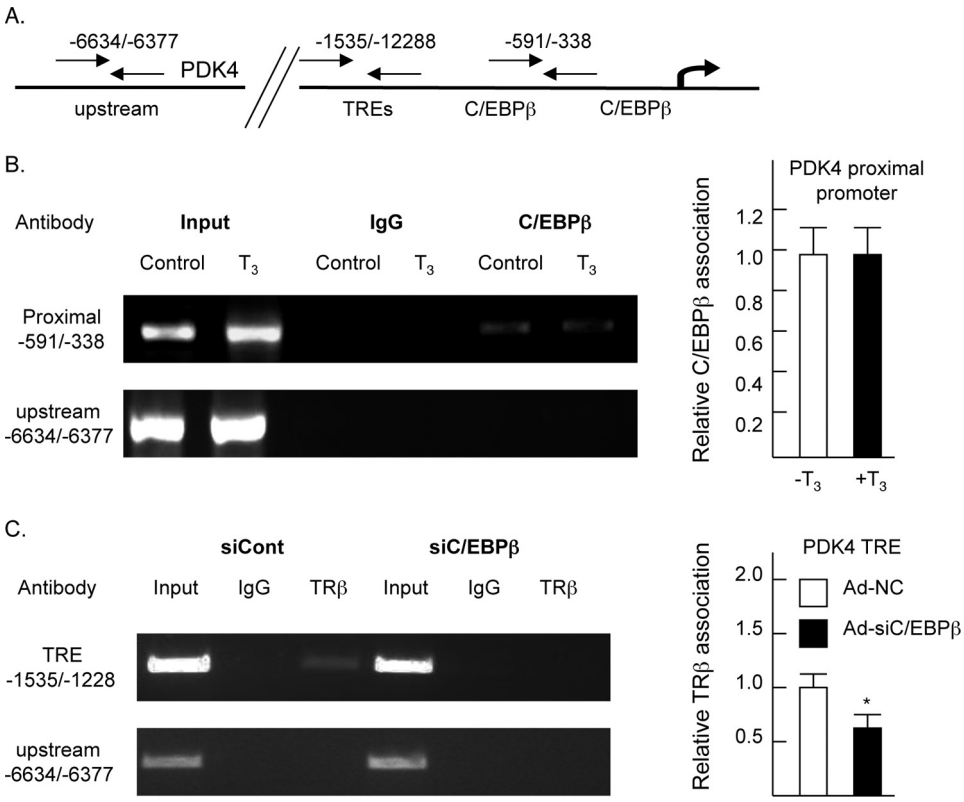

FIGURE 7. **C/EBPβ is associated with the rat *Pdk4* gene promoter *in vivo*.** *A*, a model of the *Pdk4* promoter with the locations of the ChIP primers is shown. *B*, chromatin immunoprecipitation assays were conducted on primary rat hepatocytes. Hepatocytes were treated with 100 nM T_3 for 24 h prior to cross-linking as described under "Materials and Methods." Antibodies to C/EBPβ or immunoglobulin G (IgG) were used for immunoprecipitation. The amplified PCR products using primers for the proximal and upstream regions of the rat *Pdk4* gene were resolved on agarose gels. The association of C/EBPβ with *Pdk4* proximal promoter was quantified using the Quantity One software. In the *right panel*, the data measure the relative association of C/EBPβ with ($+T_3$) or without T_3 ($-T_3$). These data are the average ± S.E. of four independent ChIP assays. *C*, ChIP assays were conducted on cells infected with adenovirus expressing Ad-NC (Ad-siControl) or Ad-siC/EBPβ. Antibodies to TRβ or IgG were used for the immunoprecipitation. The amplified products were for the TRE region of the *Pdk4* promoter. On the right, the data show the relative decrease in TRβ binding and are the average of four independent ChIP assays. *, $p = 0.01–0.05$.

knockdown of C/EBPβ decreased the binding of TRβ to the *Pdk4* promoter (Fig. 7*C*). These data suggest that C/EBPβ assists in recruitment of TRβ to the *Pdk4* gene.

DISCUSSION

T_3 controls multiple aspects of hepatic metabolism including fatty acid oxidation, lipogenesis, and glucose oxidation (47). T_3 stimulates *Pdk4* gene expression and decreases PDC activity in the heart, liver, and skeletal muscle (48–50). We recently reported that T_3 induces *Pdk4* expression through two TREs in the *Pdk4* promoter located 1,150 bp upstream of the transcriptional start site (32). Here, we investigated the role of C/EBPβ in the basal and T_3-regulated expression of the *Pdk4* gene. Our data demonstrate that C/EBPβ stimulates *Pdk4* expression. In addition, C/EBPβ enhances the T_3 induction of *Pdk4* and *Cpt1a*. Our results suggest that C/EBPβ will contribute to the hormonal regulation of a subset of hepatic genes.

In addition to activating the TR, T_3 can affect transcription by altering the abundance and activity of transcription factors and coactivators. For example, we and others found that T_3 increased the levels of the transcriptional coactivator PGC-1α (21, 34). We also observed elevated association of PGC-1α with the *Pdk4* and *Cpt1a* genes following T_3 administration (32, 34). To our knowledge, only one study has investigated the effect of T_3 on the abundance of C/EBP proteins. Menéndez-Hurtado *et al.* (23) reported that both C/EBPα gene expression and C/EBPβ gene expression were decreased in livers of pups delivered from hypothyroid rats. Administration of T_3 to the hypothyroid animals increased the hepatic C/EBPα and C/EBPβ mRNA and protein levels (23). In our studies, we observed that the addition of T_3 to hepatocytes induced C/EBPβ gene expression and protein abundance. Surprisingly, T_3 did not increase the association of C/EBPβ with the *Pdk4* gene in the ChIP assays. It seems that the mechanism by which C/EBPβ enhances T_3 responsiveness does not involve increased C/EBPβ binding. C/EBPβ participates in the glucocorticoid induction of the *Pepck* gene (12). However, increased C/EBPβ binding was not observed on the *Pepck* gene following the addition of dexamethasone (10).

Overexpression of C/EBPβ increased the expression of *Pdk4*, suggesting that C/EBPβ regulates this gene. Previous studies indicated that C/EBPβ knock-out mice had altered regulation of gluconeogenic and lipogenic genes following various dietary manipulations (8, 52). However, knocking out C/EBPβ did not reduce significantly the basal levels of *Pdk4* and *Cpt1a* mRNA in fed animals (52). The expression of PGC-1α, an important coactivator of *Pdk4* and *Cpt1a*, was reduced in C/EBPβ knock-out mice (52). We also found that *Pdk4* and *Cpt1a* mRNA levels were not decreased in the

livers from C/EBPβ knock-out mice (data not shown). These results are similar to previous reports showing that C/EBPβ is not essential for the maintenance of basal *Pepck* mRNA levels in mice (6). However, C/EBPβ deletion limited the full induction of *Pepck* and glucose-6-phosphatase genes by streptozotocin-induced diabetes (6). Our results and those of others suggest that C/EBPβ is not essential for maintaining the basal levels of *Pdk4* and *Cpt1a* but likely contributes to the hormonal regulation of these genes (6).

Earlier studies demonstrated that C/EBPβ is extensively involved in the hormonal control of the *Pepck* gene by cAMP and glucocorticoids (12, 51). In addition, the switching of the liver-enriched activator protein (LAP) and liver-enriched inhibitory protein (LIP) isoforms of C/EBPβ contributes to the insulin inhibition of *Pepck* gene expression (10). We have shown that C/EBPβ participates in the T_3 induction of the *Pepck* gene and that the transactivation domain of C/EBPβ is needed (24). Here, we used multiple approaches to demonstrate that C/EBPβ is required for the full T_3 induction of *Pdk4* and *Cpt1a*. Overexpression of C/EBPβ enhanced the T_3 induction. The dominant negative A-C/EBP diminished the T_3 induction of *Pdk4* and *Cpt1a*. We used adenoviral-mediated silencing of the C/EBPβ. The knockdown of C/EBPβ diminished the ability of T_3 to induce *Pdk4*, *Cpt1a*, and *Pepck* (data not shown) gene expression. C/EBPβ knockdown modestly impacted the induction of *Fasn*. These data indicate that C/EBPβ is an important coregulator for the T_3 induction of selected genes in primary hepatocytes. For both the *Pdk4* and the *Cpt1a* genes, the C/EBP binding sites are not adjacent to the TRE. Our ChIP data suggest that the role of C/EBPβ may be to enhance the association of TRβ with the *Pdk4* gene. In addition, the absence of C/EBPβ may impair the recruitment or abundance of coactivators. In fact, it has been found that C/EBPβ increases the expression of PGC-1α, and we have shown that PGC-1α enhances the T_3 induction of *Cpt1a* and *Pdk4*.

Based on our studies, we conclude that C/EBPβ induces the *Pdk4* gene expression through two C/EBPβ-response elements in the *Pdk4* promoter. T_3 increases the abundance of the C/EBPβ but does not increase its association with the *Pdk4* promoter. Our data suggest that C/EBPβ is an important accessory factor for the T_3 activation of several hepatic genes.

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